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(54) Title: COMPOSITIONS AND METHODS FOR THE CHARACTERIZATION AND TRANSPLANTATION OF MAMMALIAN RETINAL STEM CELLS

(57) Abstract

Methods are disclosed for generating retinal progenitor cells which may be used to advantage for retinal transplantation in patients suffering from degenerative disorders of the retina. The cells of the invention may also be used to screen beneficial therapeutic agents which promote survival and/or differentiation of retinal progenitor cells.

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COMPOSITIONS AND METHODS FOR THE CHARACTERIZATION AND TRANSPLANTATION OF MAMMALIAN RETINAL STEM CELLS

FIELD OF THE INVENTION

This invention relates to the fields of cell biology and opthamology. More specifically, the invention provides compositions and methods for the isolation and transplantation of retinal progenitor cells to correct certain degenerative disorders of the retina. Screening methods are also provided for assaying test agents for survival and differentiation promoting effects on retinal progenitor cells.

BACKGROUND OF THE INVENTION

Several publications are referenced in this application by numerals or author name in parentheses in order to more fully describe the state of the art to which this invention pertains. The disclosure of each of these publications is incorporated by reference herein.

Photoreceptors are cells of the neurosensory retina which play a pivotal role in generating the signal for vision. Degeneration of photoreceptors, whether it is inherited or age-related, is the most common cause of blindness. In both retinitis pigmentosa (RP) and macular degeneration (MD), two of the most prevalent of the sight robbing diseases, photoreceptors are the target. RP, which is a heterogenous group of inherited degenerative diseases, affects 1.5 million people worldwide and approximately 100,000 people in the USA from all walks of life. RP patients have difficulty seeing at night and their peripheral visual field is

restricted in early adulthood. With the progression of the disease, the peripheral visual field constricts so much so that by the age of 60, the majority of RP patients become legally blind. Despite the extensive genetic heterogeneity, RP primarily affects the photoreceptors.

Degenerative changes involving photoreceptors also occur in the macula, the region in the central retina which is responsible for sharp and acute vision. MD patients have difficulties performing routine tasks such as driving, reading and face recognition, thus seriously compromising their quality of life. MD is more common in advanced age; approximately 10 million Americans suffer from visual loss due to age-related macular degeneration (ARMD) and as the US population ages, more patients will become blind due to ARMD than from glaucoma and diabetic retinopathy combined. Unfortunately, there is no effective treatment for photoreceptor dystrophy encountered in RP and MD, thus adding to the emotional and financial burden on society.

The present inventor has appreciated the need for compositions and methods for reversing retinal degeneration. Such methods and compositions are provided herein.

SUMMARY OF THE INVENTION

The present invention provides compositions and methods for the generation of retinal progenitor cells suitable for transplantation in test subjects having retinal degenerative disorders.

In a preferred embodiment of the invention, multipotent retinal stem cell progenitors are provided which have the capacity to differentiate into

photoreceptors. Such cells may be used to advantage in retinal transplant procedures to prevent and/or correct retinal degenerative disorders.

The retinal stem cell progenitors of the invention may be genetically engineered to express growth factors for promoting survival of the transplanted cells. Expression of growth factors by the transplanted cells should promote graft survival in the retina. Growth factors envisioned for this purpose include, but are not limited to, EGF, bFGF, BDNF, TGFα, TGFβ, IGF and CNTF. The nucleic acid sequences encoding these growth factors are readily available and may be obtained from GenBank. Methods for introducing nucleic acids into the retinal progenitor cells of the invention include but are not limited to retroviral mediated transformation, electroporation, transfection, lipofection, and calcium phosphate precipitation.

In yet another embodiment of the invention, the primary retinal progenitor cells of the invention are immortalized using methods known to those of skill in the art. Cells may be immortalized via the introduction of nucleic acid molecules encoding the following: SV40 large T antigen, papillomavirues E6 and E7, adenovirus EIA, Epstein Barr virus, oncogenes such as myc, and mutant p53. Retinal cells so immortalized may be used to advantage in assays to assess the survival promoting effects of test agents suspected of having this activity.

A method for preparing retinal progenitor stem cells of the invention is also provided. This method entails obtaining embryonic retina explants and dissociating the explants into single cell retinalsphere suspensions. The retinalspheres are then plated and

exposed to an effective amount of a growth factor. Exposure to EGF for example gives rise to retinal cells having the sustained proliferative properties.

In an alternative embodiment, following isolation, the cells are co-cultivated with PN1 cells to promote growth and differentiation. Following isolation and clonal outgrowth the retinal progenitor cells of the invention may be cryopreserved for future use.

In another aspect of the invention, a method for determining the survival promoting effects of a test agent on retinal stem cell progenitors is provided. The method entails exposing a population of retinal stem cell progenitors to a test agent suspected of having survival promoting effects. The survival promoting effects if any, will then be assessed. Survival enhancement may be indicated by enhanced proliferation and/or prolonged survival in culture.

In yet another aspect, the invention provides a method for determining the effect of a test agent on the differentiation of retinal stem cell progenitors. In this embodiment, a population of retinal stem cell progenitors is contacted with a test agent suspected of inducing differentiation of retinal stem cells. The differentiating effects of the test agent are then assessed. Differentiation of stem cells may be analyzed using a variety of methods, including, but not limited to immunohistochemical and morphological assays, and molecular biological assays. Reagents are available for use in all of the assays described above for determining the presence or absence of retinal cell differentiation markers following exposure to the test agent.

In a preferred embodiment of the invention, a method for transplanting retinal stem cell progenitors

into a retina of a test subject is provided. Following preparation of a retinal stem cell population, the cells are resuspended in a biologically compatible medium and delivered to the retina of a test subject. In one embodiment, the cells are injected into a lesion site between the choroid of the eye and the retina, i.e. the subretinal space. The retinal stem cells so transplanted may optionally include at least one heterologous nucleic acid molecule encoding a growth factor. Retinal cells expressing growth factors may be superior in promoting graft survival following transplantation.

The following definitions are provided to facilitate an understanding of the present invention:

A "retinal stem cell progenitor" as used herein refers to a multipotent stem cell, which under the appropriate culture conditions may be induced to differentiate into photoreceptor cells.

The terms "growth factor" refers generally to multifunctional, locally acting, intercellular signaling peptides which control both the ontogeny and maintenance of tissue form and function. A variety of different growth factors may be utilized in the practice of the present invention. These include, without limitation, bFGF, EGF, TGFα, TGFβ, IGFs, and CNTF. The nucleic acid sequences encoding these factors are known and readily available to one of ordinary skill in the art.

"Nucleic acid" or a "nucleic acid molecule" as used herein refers to any DNA or RNA molecule, either single or double stranded and, if single stranded, the molecule of its complementary sequence in either linear or circular form. In discussing nucleic acid molecules, a sequence or structure of a particular nucleic acid

molecule may be described herein according to the normal convention of providing the sequence in the 5' to 3' direction.

A "replicon" is any genetic element, for example, a plasmid, cosmid, bacmid, phage or virus, that is capable of replication largely under its own control. A replicon may be either RNA or DNA and may be single or double stranded.

A "vector" is a replicon, such as a plasmid, cosmid, bacmid, phage or virus, to which another genetic sequence or element (either DNA or RNA) may be attached so as to bring about the replication of the attached sequence or element.

An "expression operon" refers to a nucleic acid segment that may possess transcriptional and translational control sequences, such as promoters, enhancers, translational start signals (e.g., ATG or AUG codons), polyadenylation signals, terminators, and the like, and which facilitate the expression of a polypeptide coding sequence in a host cell or organism.

The terms "transform", "transfect", "transduce", shall refer to any method or means by which a nucleic acid is introduced into a cell or host organism and may be used interchangeably to convey the same meaning. Such methods include, but are not limited to, transfection, electroporation, microinjection, PEG-fusion and the like.

The introduced nucleic acid may or may not be integrated (covalently linked) into nucleic acid of the recipient cell or organism. In bacterial, yeast, plant and mammalian cells, for example, the introduced nucleic acid may be maintained as an episomal element or independent replicon such as a plasmid. Alternatively,

the introduced nucleic acid may become integrated into the nucleic acid of the recipient cell or organism and be stably maintained in that cell or organism and further passed on or inherited to progeny cells or organisms of the recipient cell or organism. In other manners, the introduced nucleic acid may exist in the recipient cell or host organism only transiently.

A "clone" or "clonal cell population" is a population of cells derived from a single cell or common ancestor by mitosis.

A "cell line" is a clone of a primary cell or cell population that is capable of stable growth *in vitro* for many generations.

An "immortalized cell" is used herein to refers to a cell which will proliferate indefinitely in culture. Cells obtained from primary cultures have a finite life span. However, the introduction of nucleic acids encoding certain oncogenes or viral proteins transforms such cells such that they may be passaged indefinitely in culture. Immortalizing nucleic acids known to be effective in the generation continuous cells lines, include, without limitation, those encoding SV40 large T antigen, papillomaviruses E6 and E7, adenovirus E1A, Epstein Barr virus, human T cell leukemia virus, herpesvirus saimiri, oncogenes such as myc and mutated p53.

A "signal peptide" as used herein refers to a nucleic acid sequence that directs newly synthesized secretory or membrane proteins to and through membranes of the cell. An exemplary signal peptide of the present invention is the prepro sequence of NGF, however, other signal peptides may prove useful in the practice of the present invention. Sequences encoding such signal

peptides are known to those of ordinary skill in the art.

A "test subject" as used herein includes both humans and animals.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B are micrographs that show the growth stimulatory effects of epidermal growth factor (EGF) on retinal progenitor cells. The dividing cells, identified by the silver grains corresponding to the incorporated tritiated thymidine, were localized in the outer neuroblastic layer (Nbl) which harbors the retinal progenitors, Fig. 1A. The proportion of dividing cells (arrows) in the outer neuroblastic layer increased in response to EGF (Fig. 1B) in comparison to that in the control (Fig. 1A), suggesting that the developing retina harbors EGF-responsive progenitors similar to those found in the developing striatum (1,2). IR= Inner retina.

Figures 2A-2C are micrographs depicting retinalspheres formed in response to saturating concentrations of EGF (20 ng/ml). By the end of the first week in culture, retinalspheres of various sizes are observed (Fig. 2A; magnification x100). A higher magnification of these retinalspheres is shown in Fig. 2B (magnification, x200). These retinalspheres have been maintained in culture for 60 days in the presence of EGF. Additionally, each neurosphere can be dissociated by trituration and upon re-culture in suspension cells can form secondary retinalspheres (Figure 2C, magnification, x100) suggesting that the cells have the self-renewal property of stem cells.

Figures 3A-3C are a series of micrographs showing the proliferating progenitors in retinalspheres following immunostaining with anti-BrdU and anti-nestin. The majority of the cells in the retinalspheres incorporated BrdU suggesting that these cells were in the S-phase of the cell cycle when exposed to BrdU (Figs. 3A and 3B). The BrdU+ cells were also positive for nestin, a marker for neuroectodermal stem cells (Fig. 3B; magnification,x400) suggesting that the proliferating cells in retinalspheres possess progenitors' properties. Figure 3A is a Nomarski image.

Figures 4A-4I are a series of micrographs showing retinalspheres which were fixed and analyzed by double immunocytochemistry in the following combinations: anti-BrdU/anti-neurofilament (NfL; neuronal marker); anti-BrdU/anti O4 (oligodendrocytic markers) and anti-BrdU/anti-GFAP (astrocytic markers). The BrdU+ cells in the retinalspheres expressed the neuronal (Fig. 4A), oligodendrocytic (Fig. 4B) and astrocytic (Fig. 4C) markers suggesting that the EGF-responsive progenitors are multipotential. However, the proportion of neurofilament expressing cells was considerably higher than those expressing either O4 or GFAP. Similar results were obtained with secondary retinalspheres (data not shown). Figures 4A, 4D and 4G are Nomarski images. Magnification, x400.

Figure 5 shows the results of Southern analysis which reveal the expression of the opsin gene in retinal progenitors treated with EGF. Using RT-PCR analysis to amplify 400bp of the opsin transcript, the data show

that the expression of opsin increases significantly when EGF is withdrawn from the culture for three days. Addition of bFGH (20ng/ml) which has been shown to promote photoreceptor differentiation (3) had a moderate additive effect on opsin gene expression. The upper panel in the figure shows Southern analysis of the RT-PCR products obtained by amplifying cDNAs using opsin gene specific primers (4). The lower panel shows the ethidium bromide staining of RT-PCR products obtained by amplifying corresponding cDNAs using primers corresponding to the sequence of a constitutively expressed gene, β-actin. The RT-PCR products were resolved on 1% agarose gel by electrophoresis.

Figures 6A and 6B depict a pair of micrographs showing that co-culture with PN1 retinal cells promotes the differentiation of retinal progenitors into photoreceptors. The cells in neurosheres were fixed and subjected to double-immunocytochemistry using anti-BrdU and RetP1 (anti-opsin) antibodies. Fig. 6A is a Nomarski image. The majority of BrdU+ cells (green nuclei) were also opsin positive (red halo) suggesting that retinal co-culture conditions promote photoreceptor differentiation (Fig. 6B).

Figure 7 is a schematic diagram depicting the methodology utilized for generating retinal progenitor cells of the invention.

Figure 8 is a schematic diagram depicting the protocol for introduction of nucleic acid constructs in to retinal stem cells followed by transplantation into a test subject.

DETAILED DESCRIPTION OF THE INVENTION

Degeneration of photoreceptors, whether it is inherited or age-related, is the most common cause of blindness. Photoreceptors are cells of the neurosensory retina without which the signal for vision cannot be generated. In both retinitis pigmentosa (RP) and macular degeneration (MD), two of the most prevalent of the sight robbing diseases, photoreceptors degenerate. Unfortunately, there is no effective treatment for photoreceptor dystrophy encountered in RP and MD, thus adding to the emotional and financial burden on society. However, in both RP and MD other retinal cells are spared. Accordingly, it may be possible to restore vision by therapeutical intervention via treatment with growth factors that promote the survival of photoreceptor accompanined by retinal transplantation. If photoreceptors may be rescued from degeneration and/or replaced with the restoration of connections with unaffected neurons in the diseased retina, some recovery of visual function may be obtained.

The use of exogenous growth factors and retinal transplantation have distinct advantages as therapeutic approaches to photoreceptor dystrophy. For example survival promoting growth factor can prevent premature death of photoreceptor thus delaying the degenerative process. Retinal transplantation has the added advantage of reconstructing damage retina by providing new photoreceptors that can restore functional circuitry needed for vision.

Among the models of inherited photoreceptor dystrophy, RCS rats are the most studied for transplantation purposes (5). In these rats,

photoreceptors begin to degenerate in the third post-natal week. By the second month, the photoreceptor layer is reduced to 2-cells thick as compared to the normal retina which has an 8-10 cell thick photoreceptor layer (6). The primary defect that leads to the dystrophy lies in the RPE which cannot effectively phagocytose the shed outer segments of the photoreceptors (7,8).

In contrast to the RCS rats, the defects that cause photoreceptor dystrophy in *rd* and *rds* mice reside in the photoreceptor itself. In the *rd* mouse, one of the genes involved in the transduction of visual signals, the ß-subunit of cGMP-dependent phosphodiesterase, is mutated (9). In these animals, there is a rapid degeneration of photoreceptors; the degenerative changes begin at PN8 and are completed by the fourth post-natal week (10). Photoreceptor degeneration in *rds* mice is relatively slow compared to *rd* mice. Degeneration begins in the second postnatal week and it takes 9-12 months for most of the photoreceptors to degenerate (11). The *rds* mouse is one of the most promising rodent models of RP. In this animal, the structural gene peripherin is mutated (73,74). Mutations in this gene have been linked with autosomal dominant RP and some ARMD (78,79).

A population of cells from embryonic rat retina have been isolated which are available in virtually unlimited supply. These cells may be used for retinal transplantation to repopulate damaged and diseased retina and cryopreserved for such use in the future. The cells behave like the ancestral cells of the nervous system known as stem cells, from which neurons and supporting cells of the brain are derived. The capacity

for sustained proliferation enables not only the expansion of the cells in culture, additionally, viability is maintained for prolonged periods. Finally, and most important for the purposes of the present invention, these progenitor cells can be induced to become photoreceptors, the cells that degenerate in RP and MD. Accordingly, these cells are suitable reagents for retinal transplantation and additionally will provide novel insights of the differentiation mechanisms by which retinal progenitor cells develop into photoreceptors. These data will yield valuable information about factors which may prevent photoreceptor degeneration. Such cells may provide survival factors which can slow or prevent photoreceptor degeneration. The cultured progenitor cells of the invention possess tremendous therapeutic potential to address the problem related to photoreceptor degeneration. The progenitor cells of the invention may be genetically engineered via the introduction of heterologous nucleic acid molecules encoding growth factors with retinal cell survival promoting effects.

Similar cells isolated from a different brain region have already demonstrated their ability to repair damaged areas of the brain. It has been shown that cultured precursor cells that give rise to one type of support cells of the brain were able to repopulate the spinal cord in which injury was induced and succesfully rescue the degenerative changes. The present invention is directed to the use of cultured retinal progenitors to repopulate the retina to rescue photoreceptor degeneration in animal models of human retinal dystrophy. Based on the results presented herein, the transplanted cells should develop into photoreceptors,

integrate with the host retina and rescue photoreceptors from premature death. Additionally the compositions and methods of the present invention will provide valuable information and resources for understanding and treating RP and MD. The retinal progenitor cells of the invention will also offer a viable alternative to fetal tissue dependence since the cultured progenitors have the potential to be cryopreserved and re-expanded for future transplantation purposes.

USES OF RETINAL PROGENITOR CELLS FOR THERAPEUTIC AND DRUG SCREENING APPLICATIONS

The retinal progenitor cells of the invention may be prepared from embryonic retina obtained from mammalian test subjects. The retina explants are dissociated, and exposed to growth factors which induce the formation of retinalsphreres. Retinalspheres are proliferative and multipotent. Under the appropriate culture conditions, such retinalspheres may be induced to differentiate into photoreceptors. These cells will facilitate understanding of retinal neuron differentiation and may be used to repopulate retina that has undergone degenerative changes.

The retinal stem cells so produced may then be genetically engineered using appropriate transformation vectors. Vectors may be produced for expressing growth factors in the progenitor cells of the invention. Additionally, vectors expressing appropriate nucleic acids for immortalizing progenitor cells may be synthesized. Any molecular cloning, recombinant DNA or cell culture and transformation techniques not psecivially described are carried out by standard

methods, as generally set forth, for example in **Current Protocols in Molecular Biology**, Ausubel et al. eds., 1999, J. Wiley & Sons, NY.

The retinal progenitor cells of the invention may be used in a variety of ways having utility in research, diagnostic, therapeutic and pharmaceutical applications. Representative methods of use for the compositions of the invention are described below.

As mentioned previously, immortalizing genes may introduced into the progenitor cells of the invention to generate continuous cell lines. Such cell lines will have utility in methods for assaying test compounds for survival promoting and/or differentiation inducing effects. The survival promoting effects of such test compounds may be assessed using cell viability and proliferation assays. Differentiation may be assess by the induction of expression of differentiation markers, including but not limited to opsin, IRBP, and recoverin.

The potential utility of agents or test compounds identified using the compositions and assay methods of the invention will be broad and will include uses for therapeutic intervention and prevention of retinal degenerative disorders.

Finally, as described furtherhereinbelow, the retinal stem cell progenitors of the invention may be used for transplantation to regenerate and reconstruct dystrophic retina.

The following examples are provided to facilitate the practice of the present invention. They are not intended to limit the invention in any way.

EXAMPLE I

The following protocols were utilized in practicing the present invention.

Isolation of rat retinal progenitor cells

A. Culture and Maintenance:

Time-pregnant (E-18) Sprague Dawley rats were obtained obtained from the supplier (Sasco) and retina dissociated as previously described (41). Briefly, after determining the developmental stage of the embryo by crown rump length and external features (47) eyes will be enucleated and retina removed in Hanks buffered salt solution (HBSS) with Ca^{++} and Mg^{++}. The retina will be transferred to HBSS without Ca^{++} and Mg^{++} containing 0.25% trypsin, 1mM EDTA and 20μg/ml DNase I and incubated at 37°C for 20 minutes. Trypsin will be neutralized by washing the tissue in HBSS containing 20% FBS. Cells will be dissociated by trituration (10-15 times) in the culture medium (DMEM: F12, 1xN2 supplement (GIBCO), 2mM L-glutamine, 100u/ml penicillin, 100μg/ml streptomycin) and plated at a low density (2-3x10^3 cells/cm^2) in 24-well culture dishes or in 75m^2 T-flasks for the bulk culture in the presence of 20ng/ml of EGF (GIBCO). The culture will be maintained at 37°C in 5% CO_2. By the end of one week in culture a subset of cells grow into individual colonies which are called retinalspheres (1,2). The culture will be continued for another week by which time the individual retinalspheres attain a definite and compact spherical shape. The retinalspheres will be passaged every two weeks for prolonged expansion.

In order to label the proliferating progenitors for transplantation the retinalspheres will be exposed to BrdU (10μM) for four days. The retinalspheres will be harvested and washed extensively in retinal culture medium to remove BrdU and EGF and will be dissociated into individual cells as described above except that the incubation in trypsin wil be reduced from twenty to five minutes. The proportion of labeled progenitors to be transplanted will be assessed by plating an aliquot of the dissociated cells on poly-D-lysine coated glass coverslips and counting BrdU-incorporated cells after anti-BrdU immunocytochemistry. For the identification of the graft, cells will be labeled with Fast blue. The doubled labeled cells will be assessed for viability by Trypan blue dye exclusion test.

B. Cryopreservation:

Cryopreservtion of retinal progenitors will be carried out according to previously described methods to cryopreserve neurons from primary culture (48). A similar method has been used to succesfully cryopreserve cultured spinal cord neuroblasts (49) and embryonic retinal tissue (50). Retinalspheres, 14 days in culture (DIC), will be resuspended in fresh retinal culture medium containing 1x N2 supplement, EGF (20ng/ml) and 10% DMSO. The suspension will be transferred into Nunc tubes, capped tightly and placed in a pre-chilled styrofoam container in -80°C. The next day the frozen vial will be transferred for storage in a liquid nitrogen container. For re-expansion of progenitors, the vials will be thawed quickly at 37°C and the content will be added to pre-warmed 50 ml of retinal culture medium containing EGF with a large-bore pipet. Care will

be taken to avoid mechanical damage as cryopreserved primary neuron cells are extremely sensitive to mechanical stress (48). Handling of retinalspheres with pipet will be kept to minimum. The medium will be removed by centrifugation at 1400 rpm for 10 min. Fresh retinal culture medium will be added and retinalspheres will be dis-aggregated by gentle tapping. Cultures will be maintained for one week in the presence of EGF. Progenitors in cryopreserved retinalspheres will be analyzed *in vitro* for viability, multipotentiality and expansion, and will be tagged with BrdU and Fast blue prior to transplantation. Controls will include littermates trasplanted sub-retinally with progenitors obtained from 14 DIC retinalspheres. Analyses of the transplantation will be carried out on the criteria of size, viability, extent of lamination and integration (50).

In-vitro-characterization of the retinal progenitors for transplantation:

A. Clonal Analysis:

Each batch of retinal progenitors used for transplantation will be analyzed for clonal expansion by triturating individual primary retinalspheres into single cells and plating them in individual wells in a 96-well culture plate containing 200 μl of retinal culture medium with EGF (20ng/ml) for the generation of secondary retinalspheres.

B. Multipotentiality:

The retinalspheres will be collected in conical-bottom tubes and washed three times in the retinal culture medium to remove EGF followed by plating on plated on poly-D-lysine (50μg/ml) coated glass coverslips in a 24-well culture plate. The retinal culture medium will be supplemented with 1% FBS to promote differentiation and the culture will be continued for one week. The retinalspheres will be fixed in 4% paraformaldehyde for 15 minutes at 4°C, washed in PBS and subjected to immunocytochemical analyses using anti-neurofilament and anti-MAP2, anti-04, anti GalC, and anti-GFAP antibodies.

C. Photoreceptor Differentiation:

Incubation of progenitors with FBS following the removal of EGF results in photoreceptor differentiation. To determine the effects of host tissue on the ability of progenitors to differentiate, retinalspheres will be co-cultured with dissociated retinal cells obtained from the retina of neonatal (PN1) and 2 month old Sprague Dawley rats. BrdU tagged retinalspheres will be co-cultured on poly-D-lysine coated coverslips in 24-well plates in the presence of dissociated retinal cells ($2x10^4$ cells/well) for seven days. At the end of the incubation period, the culture will be fixed and subjected to immunocytochemical analyses using retina-specific antibodies recognizing opsin (a photoreceptor marker), syntaxin (an amacrine cell marker) and β-tubulin (a ganglion cell marker).

One of the advantages of cultured retinal progenitors is the possibility that the progenitors can be manipulated in vitro to acquire a desirable phenotype(i.e., photoreceptors) prior to

transplantation. This possibility will be tested by culturing the progenitors in presence of growth factors which have been reported to promote photoreceptor differentiation. These include bFGF (3) and retnoic acid (51). BrdU tagged progenitors will be cultured in the presence of bFGF (20ng/ml) or all-trans RA (100-500nM) for one, two and three days. At the end of each incubation period, the progentors will be washed extensively to remove growth factors and cultured for another four days in presence of 1% FBS. Cells will be fixed and subjected to imunocytochemistry using anti-opsin, anti-syntaxin and anti-β-tubulin antibodies. The experiment is likely to yield information regarding the duration of exposure for optimum photoreceptor differentiation prior to transplantation. Growth factor-exposed progenitors will be used in case post-transplantational differentiation is not satisfactory.

Prompt and precise differentiation of the transplanted retinal progenitors into photoreceptors is important for the success of transplantation. RT-PCR analysis (29) of the temporal expression of opsin gene will be performed on differentiating retinal progenitors following withdrawal of EGF. The retinalspheres will be washed in retinal culture medium as described above to remove EGF and will be cultured in suspension in the presence of 1% FBS. The retinalspheres will be collected and dissociated by trypsinization as described above and counted. Total RNA will be isolated from collected cells using Trizol (GIBCO), precipitated in presence of tRNA as a carrier, dissolved in 1XPCR-amplification buffer (10mM Tris-HCl, pH 8.0; 50mM KCl; 1.5mM $MgCl_2$) and treated with RNAse free DNAse

(Promega) for 15 minutes at 37°C to remove any genomic DNA contamination. Immediately after inactivating DNAse by incubation at 90°C, first strand cDNA will be synthesized in 1X PCR amplification buffer with random hexamer (Pharmacia) using MuMLV reverse transcriptase (GIBCO) at 42°C for 30 minutes. The cDNA reaction will be used for PCR amplification using primers to amplify the cDNA of interest and a pair of primers to amplify ß-actin cDNA as an internal control. Specific amplified products will be identified by Southern blotting. If we are able to detect specific products after fewer than 25 cycles of amplification(amplifications tend to become non linear beyond this number), then normalization with levels of ß-actin expression and number of cells used for PCR amplification can yield a semi-quantitative measure of gene expression. Amplification of the opsin transcript will be accomplished by using gene-specific forward (5'CATGCAGTGTTCATGTGGGAT3) and reverse (5'GTGAGCATGCAGTTCCGGAAC3) primers. Southern analysis will be carried out using radio-labeled rat opsin cDNA cloned in our lab (4).

Transplantation of retinal progenitors

A. Animal Models:

All animals will be maintained according to recommendations by the Department of Health and Human Services and ARVO guidelines. Sprague Dawley rats and *rd* mice will be purchased from Sassco and Charles River, respectively. A breeding pair of *rds* mice is available to us from Dr. Michael Chaitin at the University of North Texas Health Science Center, Fortworth, Texas. The animals will be housed in the animal care facility of

the University of Nebraska Medical Center at 22°C under a 12-hour light-day cycle.

To evaluate the parameters of survival, differentiation and integration of the graft transplantation will be carried out in immature (PN10) and young adult Sprague Dawley rats. Transplantation in immature and young adult host will provide information regarding host-transplant interaction with respect to the host's age. Evaluation of transplantation will be carried out at 2-, 4-, 8 weeks and after a year by morphological and morphometric analyses using light and electron microscopy, immunocytochemistry and in situ hybridization.

The *rd* mouse is a suitable model for the evaluation of retinal progenitors to repopulate and reconstruct damaged retina since photoreceptors degenerate rapidly (completed by fourth post-natal week) in this animal. Sub-retinal transplantation will be carried out in 2-3 month old *rd* mice and the transplant will be analyzed as described above at 2-, 4-, 8 weeks and 6 months post-transplantation.

The *rds* mouse is a suitable model to evaluate the potential of the retinal progenitors to rescue native photoreceptors from further degeneration since degeneration in this animal is relatively slow in comparison to that in the *rd* mouse. The most prominent of pre-degenerate characteristics of the rds retina is the lack of outer segments (11). The outer nuclear layer of the rds mouse has normal thickness until the age of 14 days. However, a rapid reduction in the thickness of the outer nuclear layer takes place between 14 and 21 days of age. Thereafter the rate of degeneration decreases and takes 9-12 months to complete. Sub-retinal

transplantation will be carried out one week before the onset of degeneration (PN6) and around the time of the onset of degeneration (PN14). Analyses of transplantation will be carried out as previously described at 1-, 2-, 4-, 8 weeks and 6 months post-transplantation.

B. Sub-retinal Transplantation (Trans-scleral Approach):

The transplantation protocol will be carried out in collaboration with Dr. James Turner (52). Rats and mice will be anesthetized with Ketamine (100 mg/kg bodyweight) and xyazine (5 mg/kg bodyweight). An incision will be made through the superior eye lid to expose the dorsal surface of the eye. The eye will be retracted anteriorly and inferiorly exposing the full extent of the superior rectus muscle. The muscle will be cut at its anterior attachments and reflected posteriorly exposing the sclera. On the mid-dorsal surface, using the two large superior vorticose veins as landmarks, a small penetrating lesion (0.5-1.0 mm) will be made through the sclera with a microblade. The incision will be made perpendicular to the anterior posterior axis along the equatorial plane of the eye. Penetration of the sclera to the level of the choroid will be indicated by slight bleeding at the incision site. With one additional cut, the choroid will be penetrated and the retina exposed. A 32-gauge blunt-tipped needle attached to 10μl Hamilton syringe will be held parallel to the surface and inserted with the beveled edge facing sideways, into one corner of the lesion site between the choroid and retina. The

putative stem cells (60,000-100,000) in 1μl volume will be injected. The incision will be closed with a 10-0 suture.

C. Controls:

To evaluate the effect of retinal progenitor transplantation several control experiments will be performed. This becomes particularly important in view of the fact that sham injection in the retina have been reported to cause short-term photoreceptor survival in the RCS rat (53,54,55,56). Control experiments will be performed on littermates of the experimental group. For each time-point of the analysis of transplantation, 5-7 animals will be injected with retinal progenitors in one eye and an equal number of littermates will receive vehicle (culture medium). Additional controls will include Sprague Dawley rats which will receive equal number of 14-day old identically labeled progenitors.

D. Evaluation of Transplants.

Transplanted cells will be considered graft if the following criteria (45) are fulfilled. The cells should exist as ectopic cell clusters in the sub-retinal space in serially sectioned eyes. Each cluster has more than ten cells and are not formed by macrophages, plasma cells and lymphocytes. Cells in cluster have Fast blue fluorescence and are positve for BrdU labeling. These cells will be screened for pyknotic and disintegrating nuclei to distinguish degeneration.

E. Immunosuppression for rat retinal progenitors xenograft into rd and rds mice sub-retinal space.

While we anticipate good immune tolerance by the host for allograft of the progenitors based on previous sub-retinal transplantation of embryonic cells (36), immune tolerance could be a potential problem for rat retinal progenitor xenografts in *rd* and *rds* mice. This problem can be circumvented by immunosuppression. It has been reported that xenograft of human fetal neural retina survive and are well tolerated in the sub-retinal space of cyclosporine-immunosuppressed rats (39). To immunosuppress the host (*rd* and *rds* mice) to accept rat xenografts the animals will receive daily intramascular injections of cyclosporine (10mg/kg) (22). If host tolerance of graft remains a problem after immunosuppression, retinal progenitors will be isolated from embryonic mouse retina for allograft.

Identification of transplanted retinal progenitors

Fast Blue Labeling

A rough estimation of the site of transplantation will be made by the position of the suture in the enucleated eyes (19). Fast blue fluorescence will be used to identify the graft (36). Prior to transplantation dissociated BrdU labeled-cells will be incubated in 0.025% (w/v) solution of fast blue (Sigma) in culture medium for 30 minute at 4°C. The cells will be washed in culture medium to remove fast blue and

subjected to Trypan blue dye exclusion test. We expect 80 to 98% viability following fast blue staining (36).

BrDu Labeling

In order to identify the progenitors in the graft, retinalspheres will be treated with BrdU (10μm/ml) for four days. The prolonged exposure to BrdU ensures maximal labeling since cells with stem cell-like properties have extended cell-cycle. The labeled retinalspheres will be washed extensively in culture medium prior to dissociation. Cells will be analyzed for viability by Trypan blue dye exclusion test. Additionally, a portion of cells will be plated on poly-d-lysine (50μg/ml)submerged coverslips and subjected to anti-BrdU immunocytochemistry to estimate the percentage of proliferating cells. BrdU incorporation is a reliable method of identifying transplanted cells in retina (57) and in other brain regions (58,59). Since host's photoreceptors are post-mitotic they will not incorporate BrDu and therefore can be easily distinguished from the differentiated transplants.

Light Microscopic Analysis

At appropriate post-transplantation days, animals will be anesthetized with ketamine (100 mg/kg bodyweight) and xylazine (5 mg/kg bodyweight) and eyes will be enucleated and fixed in 4% paraformaldehyde for five hours at 4°C. The eyes will be trimmed around the site transplantation (identified by the position of the suture), cyroprotected in 30% sucrose overnight at 4°C,

frozen in OCT and kept at 80°C until use. Sections for morphological and immunocytochemical analyses will be screened for the presence of the grafted progenitors by fast blue fluorescence using a Leitz A filter cube and DMR microscope

A. Morphological Analysis

For the analysis of the morphological features, measurement of the outer nuclear layer and the outer segments, 6-8μM-thick sections will be cut on a cryostat and stained with hemotoxylin & eosin. Similar analyses will be carried on 1μm-thick sections obtained for electron microscopy and stained with tolouidine blue. The retina will be divided into six sectors of equal length; superior peripheral, equatorial, and central, inferior central, equatorial and pripheral, respectively (38). The images will be digitized using Hitachi CCD camera and morphomteric analyses will be carried out using Image-Pro plus (Media Cybernetics, MD) software. For each sector three to five measurements will be made of ONL thickness and number of nuclei.

B. Immunocytochemical Analysis:

Immunocytochemical analyses (60,61) will be carried out to identify and analyze the differentiated transplanted retinal progenitors. Similar analyses will be carried out for the characterization (clonal expansion, multipotentiality and ability to differentiate into photoreceptors) of the retinalspheres prior to transplantation. Paraformaldehyde-fixed retinalspheres, dissociated cells, or tissue sections will be blocked for 30 minutes at RT in 5% serum

(depending upon the species in which the secondary antibody is raised) diluted in PBS containing 0.2% triton X-100. The sections will be washed in PBS and incubated at 4°C overnight in primary antibody diluted appropriately (See Table I) in PBS containing 0.2% Triton X-100. The following day sections will be washed in PBS and incubated for one hour at RT in cy3 conjugated secondary antibody. Sections will be washed in PBS and will be subjected to BrdU immunocytochemistry. Sections will be incubated at 37°C for 45 minutes in 2N HCL to denature DNA followed by a ten minute incubation at RT in 0.1M boric acid. Sections will be washed in PBS and BrdU immunocytochemistry will be carried out as described above. The secondary antibody used to visualize BrdU labeling will be will be conjugated with FITC. The sections will be mounted in fluoromount and viewed with Leitz-DMR microscope. The primary antibodies will also be detected, when necessary, using biotinylated secondary antibodies using ABC Vectastain (Vector) kit.

TABLE 1

Antibodies

1.RAT 401 (Developmental Studies Hybridoma Banks)	Neuroectodermal stem cells, anti-nestin
2.Mouse monoclonal anti-Neurofilament (Sigma)	Neurons
3.Mouse monoclonal anti-MAP2 (Boehringer Manheim)	Neurons
4.Rabbit antiserum to GFAP (Sigma)	Astrocytes
5.Mouse monoclonal anti O4 (Boehringer Manheim)	Oligodendrocyte
6.Mouse monoclonal anti GALC (Boehringer Manheim)	Oligodendrocytes
7.Mouse monoclonal β-tubulin (Sigma)	Retinal Ganglion Cells
8.Mouse monoclonal RET P1	Rod Photoreceptor, anti-opsin
9.Mouse monoclonal HPC-1	Amacrine cell, anti-syntaxin
10. Rabbit anti-serum to CRALBP	Muller glia

All these antibodies have been tested for cross-reactivity with rat tissues.

RETP-1 and HPC-1 are available to us from Dr. C.J. Barnstable (62) and anti-CRALBP from Dr. J. Saari (63).

C. In Situ Hybridization

To analyze the spatial aspects of photoreceptor differentiation by identification of transcripts corresponding to photoreceptor-specific genes in situ, hybridization will be carried out on transplanted and control eyes. Briefly, 8-10 μm cryostat-sections of post-fixed (4% paraformaldehyde in phosphate buffer saline) retina will be treated with proteinase K (2μg ml^{-1} proteinase K in 500 mM NaCl, 10mM Tris-HCL, pH 8.0) for 10 minutes at room temperature. After a 10 minute rinse in 0.5 XSSC at room temperature, each section will be covered with 10 μl of prehybridization buffer (50% formamide; 300 mM NaCl; 20mM Tris HCL, pH 8.0; 5 mM EDTA; 1XDenhardt; 10% dextran sulfate; 10 mM DTT) and the slides will be placed in a box saturated with 4 X SSC and 50% formamide. After an incubation at 50°C for 2 hours, 20 μl of prehybridization buffer containing heat denatured ^{35}S-labeled riboprobe (3 x 10^5 cpm) and tRNA (2.5mg ml^{-1}) will be added to each section and incubation continued at 50°C overnight. Sections will be treated in RNASE A solution (20μg ml^{-1} RNase A in 500 mM NaCl, 10mM Tris-HCl, pH 8.0) at room temperature for 30 minutes. Sections will be washed several times, with the final wash in 0.1 x SSC, 10mM ß-mercaptoethanol, 1mM EDTA at 50°C for 2 hours and then dehydrated in graded concentrations of ethanol containing 300 MM ammonium acetate. After emulsion-autoradiography, sections will

be counterstained in eosin-hematoxylin. The transplant will be analyzed for the expression of three different photoreceptor-specific genes: opsin, cGMP-gated channel and a-transducin.

Electron Microscopy

Synaptic formation, the organization, and the morphology of the outer segments at the ultrastructural level will be evaluated by electron microscopy in transplanted and control eyes to determine the differentiation and the extent of integration of transplanted cells into the host retina. Enucleated eye will be fixed in Karnovsky fixature for 30 minutes. The anterior segment will be cut at the ora serrata to facilitate diffusion and fixation continued overnight 4°C. The eyes will be post fixed in 7% osmium tetroxide, stained with lead citrate, dehydrated through an alcohol series and embedded in Epon. Thick sections (1 μM) will be cut with diamond knife, placed on copper mesh, carbon coated and photographed with the Phillip Electron Microscope.

The isolation and characterization of EGF-responsive retinal progenitors with stem cell properties serves two broad purposes. First, the cultured retinal progenitors can be utilized as the reagent for retinal transplantation. This is significant since a phase I clinical trial of fetal retinal transplantation in RP patients has already begun. The present invention may alleviate the problems associated with the scarcity of fetal tissues. Second, retinal progenitor cells in culture facilitate the

identification of epigenetic cues that regulate the specification of photoreceptors, yielding valuable information about factors which may be helpful in preventing or slowing degeneration that occurs in RP and MD (18).

It has been shown that exogenous growth factors such as bFGF, BDNF and CNTF reduce photoreceptor degeneration in inherited or light induced dystrophic retina (53). However, the exogenous growth factors may not be suitable for long term treatment due to the blood-retinal barrier and may cause pathological changes such as neovascularization. Because diffusible factors have been shown to mediate cell differentiation in the retina cultured retinal progenitors are candidates for elaborating factors that may promote survival. In addition, transplanted progenitors have the potential to repair neural tissues that have undergone degenerative changes by generating site-specific neurons. Therefore cultured retinal progenitors with stem cell properties possess tremendous therapeutic potential.

RESULTS

Identification of EGF-resposive progenitors in the embryonic rat retina

To identify progenitors in the developing retina that proliferate in response to EGF, embryonic day 18 (E18) retinal explants were grown in retinal medium in the presence of EGF (20ng/ml) for four days. In the last 24 hours of culture tritiated thymidine (1μci/ml, NEN) was added to identify proliferating cells in the S-phase of the cell-cycle. The explants were fixed, cryo protected, cryo-sectioned and subjected to emulsion

autoradiography. The dividing cells, identified by the silver grains corresponding to the incorporated tritiated thymidine, were localized in the outer neuroblastic layer (Nbl) which harbors the retinal progenitors (Figure 1). The proportion of dividing cells (arrows) in the outer neuroblastic layer increased in response to EGF (Figure 1B) in comparison to that in the control (Figure 1A), suggesting that the developing retina harbors EGF-responsive progenitors similar to those found in the developing striatum (1,2). IR= Inner retina.

The EGF-responsive progenitors can be isolated and clonally expanded

In order to isolate the EGF-responsive progenitors from the outer neuroblastic layers of the developing retina, E18 retina was dissociated (61) and cultured in suspension in the presence of a saturating concentration of EGF (20ng/ml). A subset of cells in the culture undergo rapid and extensive proliferation forming spheres termed retinalspheres. By the end of the first week in culture, retinalspheres of various sizes are observed (Figure 2A; magnification x100). A higher magnification of these retinalspheres is shoen in Figure 2B (magnification, x200). We have maintained these retinalspheres in culture for 60 days in the presence of EGF. Additionally, each neurosphere can be dissociated by trituration and upon re-culture in suspension cells can form secondary retinalspheres (Figure 2C, magnification, x100) suggesting that the cells have the self-renewal property of stem cells.

Cells in the retinalspheres are mitotic and express the stem cell marker, nestin.

In order to detect the proliferating progenitors in retinalspheres, the retinalspheres were subjected to anti-BrdU and anti-nestin immuocytochemistry following a 48-hour incubation in the culture medium containing nucleotide analog BrdU (10μM). The majority of the cells in the retinalspheres incorporated BrdU suggesting that these cells were in the S-phase of the cell cycle when exposed to BrdU (Figure 3A and 3B). The BrdU$^+$ cells were also positive for nestin, a marker for neuroectodermal stem cells (Figure 3B; magnification,x400) suggesting that the proliferating cells in retinalspheres possess progenitors' properties. Figure 3A is a Nomarski image.

The EGF responsive progenitors are multipotential.

Multipotentiality is one of the properties of progenitors with stem-cell like features. The ability of EGF-responsive retinal progenitors to differentiate along multiple lineages was tested by analyzing the phenotypes of the dividing cells following the withdrawal of EGF. Fourteen day old retinalspheres in culture medium containing EGF were incubated for 48 hours with BrdU(10μm/ml). The retinalspheres were washed extensively to remove EGF completely and the culture was continued without EGF for seven days in presence of 1% FBS. The retinalspheres were fixed and double immunocytochemical analyses were carried out in the following combination: anti-BrdU/anti-neurofilament

(NfL; neuronal marker); anti-BrdU/anti O4 (oligodendrocytic markers) and anti-BrdU/anti-GFAP (astrocytic markers). The BrdU+ cells in the retinalspheres expressed the neuronal (Figure 4A), oligodendrocytic (Figure 4B) and astrocytic (Figure 4C) markers suggesting that the EGF-responsive progenitors are multipotential. However, the proportion of neurofilament expressing cells was considerably higher than those expressing either O4 or GFAP. Similar results were obtained with secondary retinalspheres (data not shown). Figures A, D and G are Nomarski images. Magnification, x400.

The EGF-responsive progenitors can differentiate into photoreceptors.

One of the prerequisites for the utilization of the retinal progenitors for transplantation into dystrophic retina is the ability of the progenitors to differentiate into photoreceptors. This hypothesis was tested by examining opsin expression in the EGF-responsive progenitors following EGF withdrawal in E-18 explant culture. The onset of opsin expression is a reliable marker of photoreceptor differentiation (4). Using RT-PCR analysis to amplify 400bp of the opsin transcript, we observed that the expression of opsin increases significantly when EGF is withdrawn from the culture for three days (Figure 5). Addition of bFGH (20ng/ml) which has been shown to promote photoreceptor differentiation (3) had a moderate additive effect on opsin gene expression.

The upper panel in the figure shows Southern analysis of the RT-PCR products obtained amplifying cDNAs using opsin gene specific primers (4). The Lower panel shows the ethidium bromide staining of RT-PCR products obtained by amplifying corresponding cDNAs using primers corresponding to the sequence of a constitutively expressed gene, β-actin. The RT-PCR products were resolved on 1% agarose gel by electrophoresis.

Co-culture with PN1 retinal cells promotes the differentiation of retinal progenitors into photoreceptors.

We are using co-culture conditions to understand the host-transplant interactions *in vitro*. The BrdU tagged retinalspheres were co-cultured with dissociated PN1 retinal cells (10^4 cells/cm^2) for seven days in absence of EGF. The cells in retinalspheres were fixed and subjected to double-immunocytochemistry using anti-BrdU and RetP1 (anti-opsin) antibodies. A majority of BrdU+ cells (green nuclei) were also opsin positive (red halo) suggesting that retinal co-culture conditions promote photoreceptor differentiation (Figure 6A and 6B). Our preliminary observations suggest that more cells in retinalspheres differentiate into photoreceptors when in co-culture than in 1% FBS alone (data not shown). Figure 6A is a Nomarski image.

Figure 7 shows a schematic diagram which depicts the culturing methods of the invention.

EXAMPLE II

The use of exogenous growth factors and retinal transplantation have distinct advantages as therapeutic approaches to photoreceptor dystrophy. For example survival promoting growth factors may prevent premature death of photoreceptors, thus delaying the degenerative process. Retinal transplantation has the added advantage of reconstructing damaged retina by providing new photoreceptors that can restore functional circuitry needed for vision. In the present example, a combination approach to prevent photoreceptor degeneration is described. Genetically engineer retinal progenitors, which express survival-promoting growth factor for photoreceptors, bFGF, will be transplanted into dystrophic retina. bFGF is involved in the differentiation of photoreceptors and vitreal and subretinal injection of bFGF has been shown to delay photoreceptor degeneration in diseased and light damaged retina. The genetically engineered progenitors should act like a pump, providing bFGF to the damaged photoreceptor on a continuous basis thereby sustaining their survival over a prolonged period. Since bFGF has been shown to be protective to cells that make it, it should promote survival of the graft also. In addition, the bFGF expressing progenitors may also facilitate the reconstruction of damaged retina by differentiating to new photoreceptors as these progenitors have the capacity to become photoreceptor-like given the right micro-environment.

Transplantation of genetically engineered progenitors offers a multifaceted approach to treat photoreceptor degeneration encountered in sight robbing diseases. In addition, the approach described herein

enables the genetically engineered progenitor to serve as as a vehicle for delivering other growth factors or cytokines to the diseased or damaged retina.

Construction of recombinant retrovirus vectors. Cultured retinal progenitors as described in Example I, will be transduced to express bFGF by retrovirus-mediated gene transfer. Retrovirus-mediated gene transfer is preferred over adenovirus-mediated gene transfer because (1) retinal progenitors are proliferating cells and retrovirus infects dividing cells with higher efficiency; (2) the recombinant retrovirus carrying bFGF transgene will integrate stably in host cell genome; and (3) recombinant retrovirus has been used to overexpress NeuroD, a retinal differentiation factor, to facilitate differentiation of rod photoreceptors. cDNA corresponding to mouse bFGF is subcloned into retroviral vector pSRaMSVtkneo (pSRa) (Muller et al. 1991, Mol. Cell. Biol. 11:1785-1792). Transcription of bFGF cDNA is driven by 5' viral long term repeat (LTR). The presence of the neomycin resistance gene enables the selection of transduced cells in the presence of G418. Two different recombinant retrovirus constructs will be made. The first construct will be engineered to express bFGF without signal peptide (bFGF-C) and the second construct will be engineered to contain a signal peptide (bFGF-S) so that it may be secreted from the cell. In its native form bFGF does not contain the signal peptide and associates with cytosolic and the membrane fractions of the cells. In vitro and *in vivo* studies demonstrate that bFGF-C exerts greater biological activity than bFGF-S; 96% Vs 69% in promoting the survival of hippocampal neuron

(Takayama 1995, Nature Med. 1:53-58). However, bFGF-S may be more effective in rescuing photoreceptor degeneration as it should function over a longer distance. In order to secrete bFGF, a cDNA sequence corresponding to the prepro sequence of NGF will be operably linked to bFGF cDNA at 5'end. This recombinant sequence, which has been shown to encode a biologically active form of bFGF, will be cloned into pSRa (Ray et al., 1995, J. Neuro. Chem. 64:503-513). Recombinant retrovirus will be produced in 293T cells.

Selection of clones with stably integrated bFGF transgenes and analysis of expression and biological activity bFGF

In order to culture retinal progenitors E17 rat retina will be dissociated into single cells and cultured in the presence of EGF (20ng/ml) as described in Example I. Under these conditions, a subset of cells (progenitors) survive and proliferate to generate spheres of cells called retinal-spheres. The retinal spheres will be infected with recombinant retrovirus containing bFGF transgenes, bFGF-C or bFGF-S. Retinal spheres carrying recombinant retrovirus will be selected by culturing in the presence of the G418 (400ug/ml). The expression of the transgene in progenitors will be ascertained by (1) RNAse protection assay (2) immunoprecipitation of cell lysate (3) radioimmunoassay (RIA) carried out on conditioned medium using bFGF antibody (Santa-Cruz). The biological activity of bFGF-C and bFGF-S will be determined by their ability to promote survival of hippocampal neurons in a co-culture condition as described previously (Ray et al., 1995,

supra). Controls will include non-transduced progenitors.

Differentiation of Progenitors into Photoreceptors following introduction of bFGF transgenes.

Besides their utilization as a reagent for the continuous delivery of survival-promoting growth factor, bFGF, the ability of genetically engineered progenitors to differentiate to photoreceptors will also be determined. Cultured progenitors, when co-cultured with neonatal retinal cells display photoreceptor-specific properties. The BrdU tagged-genetically engineered cells will be co-cultured with PN1 retinal cells for 4-5 days and analyzed for expression for photoreceptor-specific markers such as the opsin, recoverin and IRBP.

Promotion of photoreceptor survival in vitro following introduction of bFGF transgenes into progenitors.

Prior to transplantation of progenitors with bFGF transgene, promotion of photoreceptor survival will be tested in vitro. Retinal explants obtained from RCS rat, *rd* and *rds* and control animals will be cultured in the presence of bFGF-transduced retinal progenitors. Explants will be dissociated into single cells and the proportion of cells expressing photoreceptor-specific marker and those displaying apoptotic changes will be determined using immunocytochemical analysis and Tunel method (Chang et. al., 1993, Neuron 11:595-605), respectively.

Transplantation of genetically engineered retinal progenitors with bFGF transgenes in animal models of photoreceptor dystrophy

The ability of genetically engineered progenitors to rescue and/or promote survival of photoreceptors will be assessed in three different models (RCS rat, *rd* mouse and *rds* mouse) of photoreceptor dystrophy. The genetic defects and the temporal aspects of degeneration are distinct in these models. bFGF has been shown to slow retinal degeneration in RCS rats and rd mice and its effect in rds mice has not been evaluated. Sub retinal transplantation will be carried out by trans-scleral approach. Approximately 60,000 to 100,00 genetically engineered cells will be injected into the retina in a 1 μl volume. The outcome of the transplantation will be evaluated by morphological, morphometric, immunocytochemical and in situ hybridization analyses, and also by Tunel method to evaluate programmed cell death (Chang et al., 1993, supra).

Since bFGF will be available to the damaged photoreceptors on continuous basis their survival should be sustained for a longer period of time than those observed after vitreal or subretinal injection of exogenous bFGF. A schematic diagram depicting the protocol to be utilized in the practice of Example II is provided in Figure 8.

While delivery of bFGF retinal progenitor cells has been exemplified herein, other growth factors may be utilized in the present invention. The survival and or differentiating effects of these additional growth factors will be assessed using the criteria described herein. Growth factors contemplated for use in the

invention include, but are not limited to EGF, TGF, CNTF, IGF, and the like.

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While certain of the preferred embodiments of the present invention have been described and specifically exemplified above, it is not intended that the invention be limited to such embodiments. Various modifications may be made thereto without departing from the scope and spirit of the present invention, as set forth in the following claims.

What is claimed is:

1. A multipotent retinal stem cell progenitor.

2. The retinal stem cell progenitor of claim 1, further comprising at least one heterologous nucleic acid encoding a growth factor.

3. The retinal stem cell progenitor of claim 2, wherein said growth factor is selected from the group consisting of EGF, bFGF, BDNF, TGFα, TGFβ, CNTF, and IGF.

4. The retinal stem cell progenitor of claim 2, wherein said at least one heterologous nucleic acid is introduced into said cells via a process selected from the group consisting of retroviral mediated transformation, electroporation, transfection, lipofection, and calcium phosphate precipitation.

5. An immortalized retinal stem cell progenitor of claim 1.

6. A method for preparing retinal progenitor stem cells comprising:

a) obtaining embryonic retina explants;

b) dissociating said embryonic retina explant in to a single cell retinalsphere suspension and plating said retinalspheres;

c) exposing said retinalspheres to a growth factor such that the cells remain proliferative and uncommitted; and

d) isolating said retinal progenitor cells.

7. The method of claim 6, wherein said retinal progenitor cells are further co-cultured with PN1 retinal cells.

8. The method of claim 6, wherein said growth factor is selected from the group consisting of EGF, bFGF, BDNF, TGFα, TGFβ, CNTF, and IGF.

9. The method of claim 6, wherein following isolation of said retinal progenitor cells, said cells are cryopreserved.

10. A method for determining the survival promoting effects of a test agent on a retinal stem cell progenitor, comprising:

a) providing a population of retinal stem cell progenitors;

b) contacting said progenitors with a test agent suspected of having survival promoting effects;

c) determining said survival promoting effects of said test agent.

11. A method as claimed in claim 10, wherein said survival promoting effects are determined by an assay selected from the group consisting of MTT assay, and trypan blue exclusion assay.

12. A method for determining the effect of a test agent on the differentiation of retinal stem cell progenitors, comprising:

a) providing a population of retinal stem cell progenitors;

b) contacting said progenitors with a test agent suspected of inducing differentiation;

c) determining the differentiating effects of said test agent.

13. A method as claimed in claim 12, wherein said differentiation of retinal stem cell progenitors is determined by determining the expression of markers selected from the group consisting of opsin, recoverin, IRBP, syntaxin, beta tubulin, mGLUR6, PKC, oligodendrocytic markers, astrocytic markers, neuronal and retinal phenotype specific markers

14. A method for transplanting retinal stem cell progenitors into a retina of a test subject, comprising:

a) providing a retinal stem cell progenitor population; and

b) delivering said cells to the retina of a test subject.

15. A method as claimed in claim 14, wherein said cells are delivered to said test subject by injecting the cells into a lesion site between the choroid of the eye and the retina.

16. A method as claimed in claim 14, wherein said retinal stem cell progenitors comprise at least one heterologous nucleic acid encoding a growth factor.

17. A method for transplanting retinal stem cell progenitors into a retina of a test subject, comprising:

a) obtaining and dissociating an embryonic retina, thereby generating a retinalsphere population;

b) exposing said retinalspheres to at least one growth factor, said growth factor inducing proliferation of said retinalspheres;

c) dissociating said retinalspheres;

d) resuspending said dissociated retinalspheres in a biologically compatible medium; and

e) injecting said dissociated retinalspheres into a retina of a test subject.

18. A method as claimed in claim 17, wherein following the dissociation in step c), the cells are contacted with growth factor to maintain proliferative capacity.

19. A method as claimed in claim 17, wherein following the dissociation in step c), the cells are contacted with a growth factor which induces differentiation.

20. A method as claimed in claim 17, wherein said retinal stem cell progenitors comprise at least one exogenous nucleic acid encoding a growth factor.

Fig. 1A

Fig. 1B

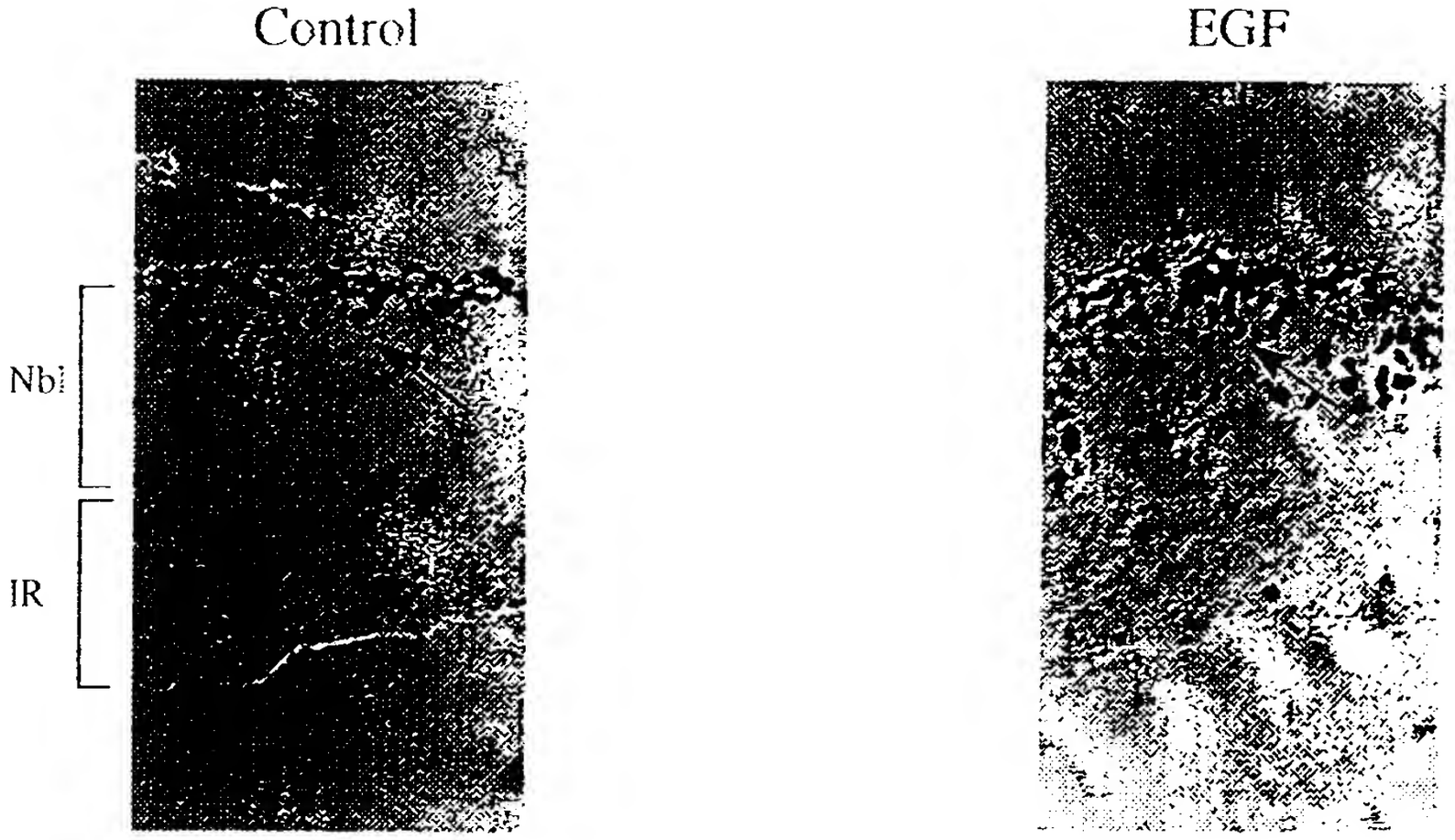


Fig. 2A

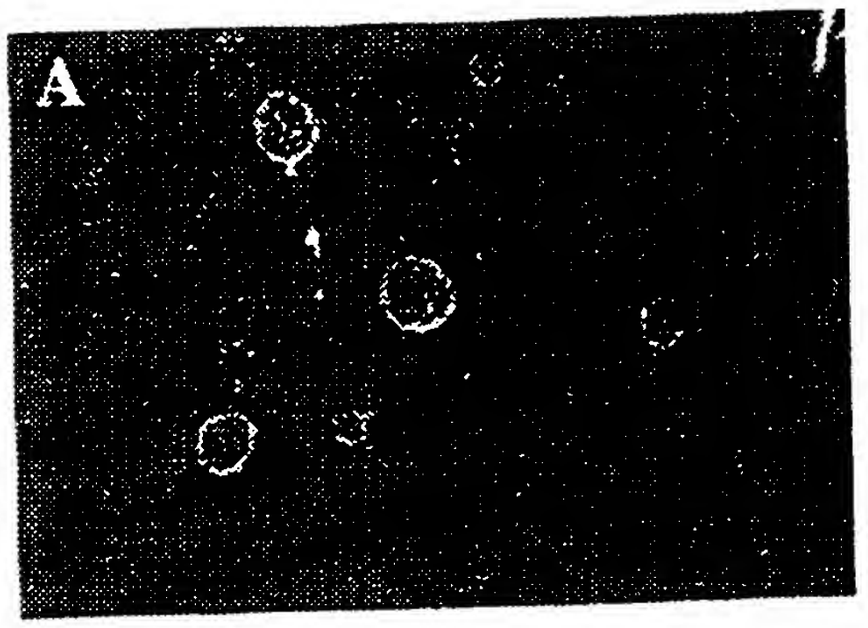


Fig. 2B

Fig. 2C

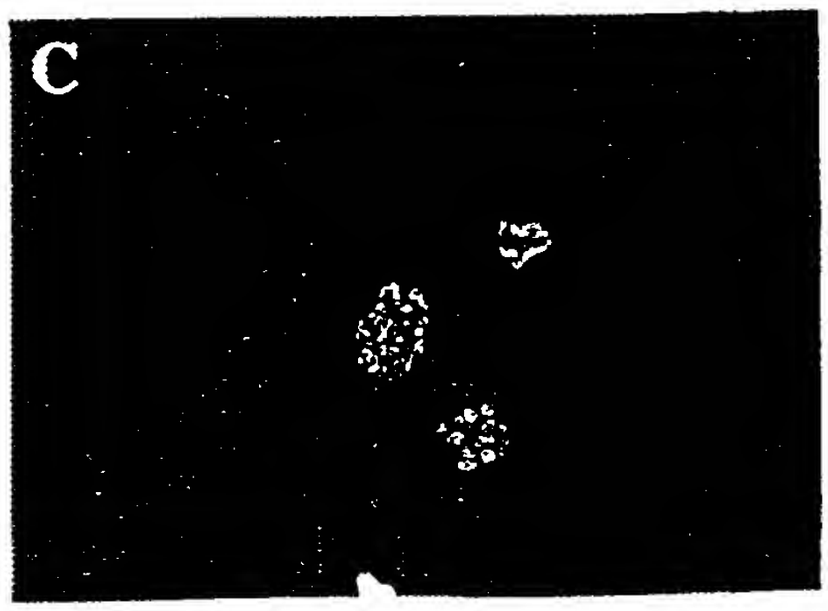


Fig. 3A

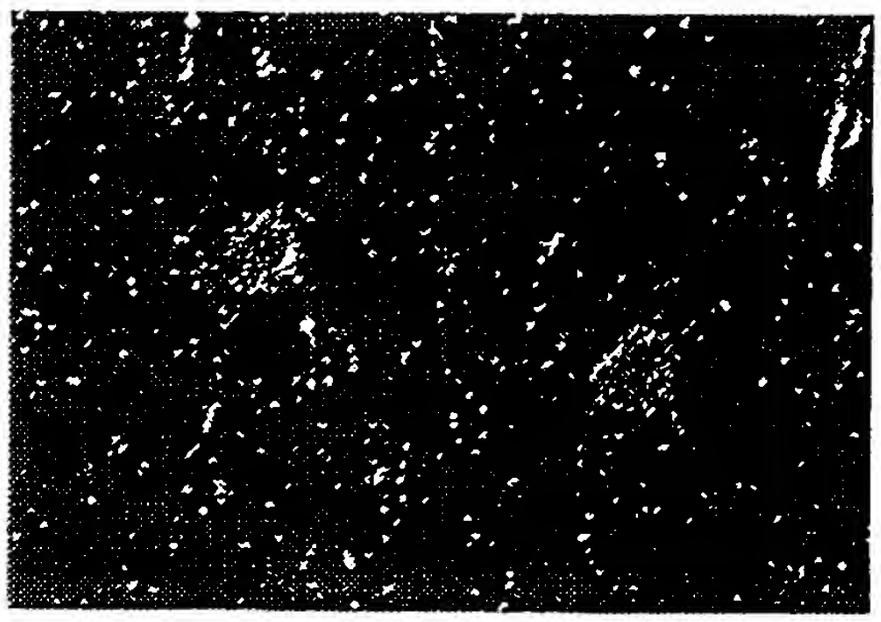


Fig. 3B

Fig. 3C

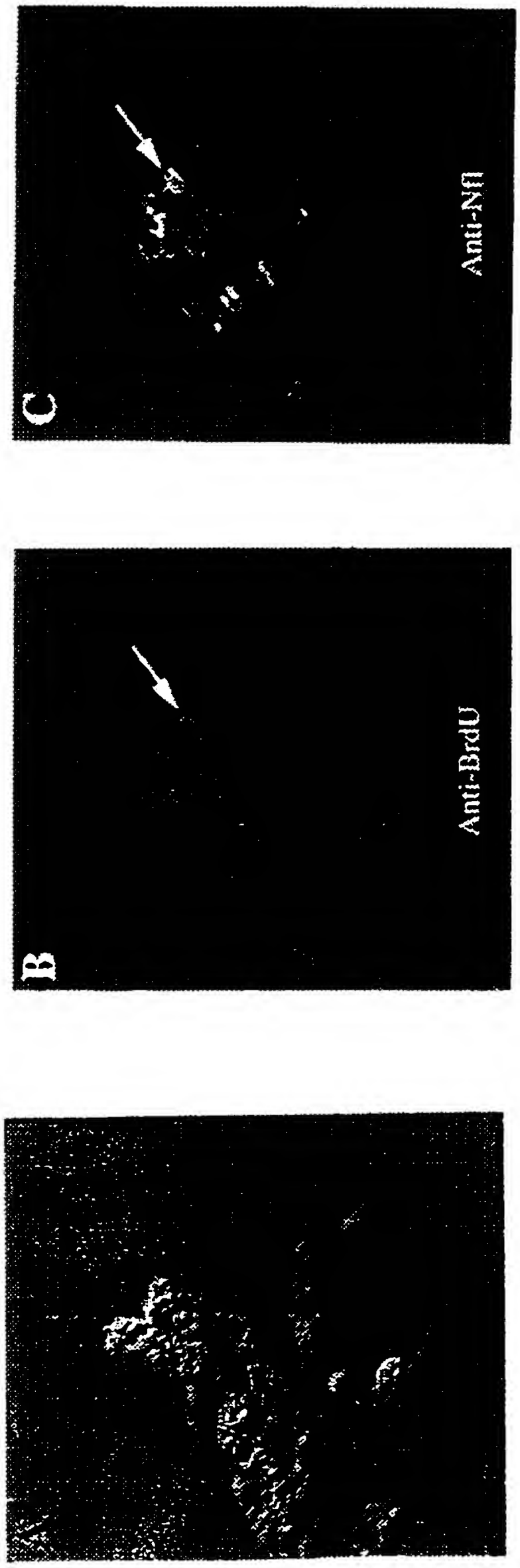


Fig. 4C

Fig. 4B

Fig. 4A

Fig. 4D

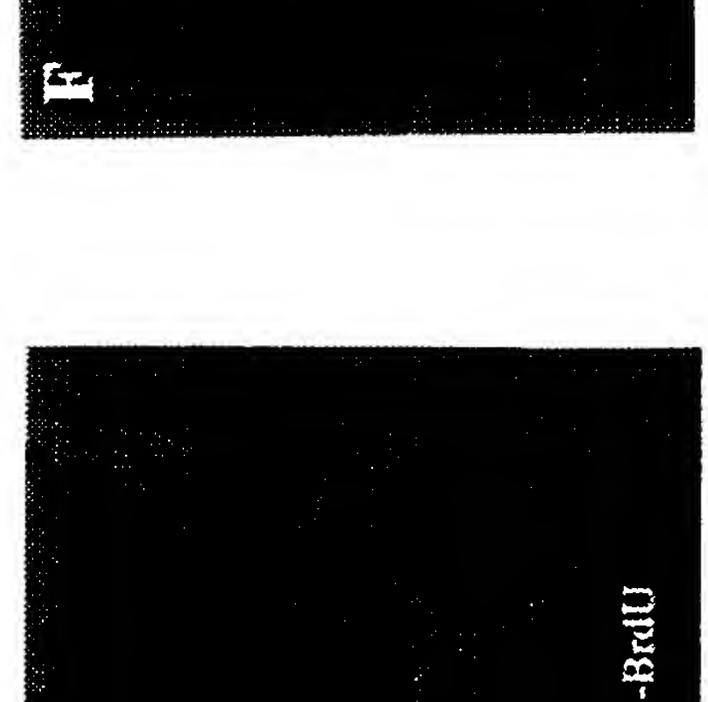


Fig. 4E

Fig. 4F

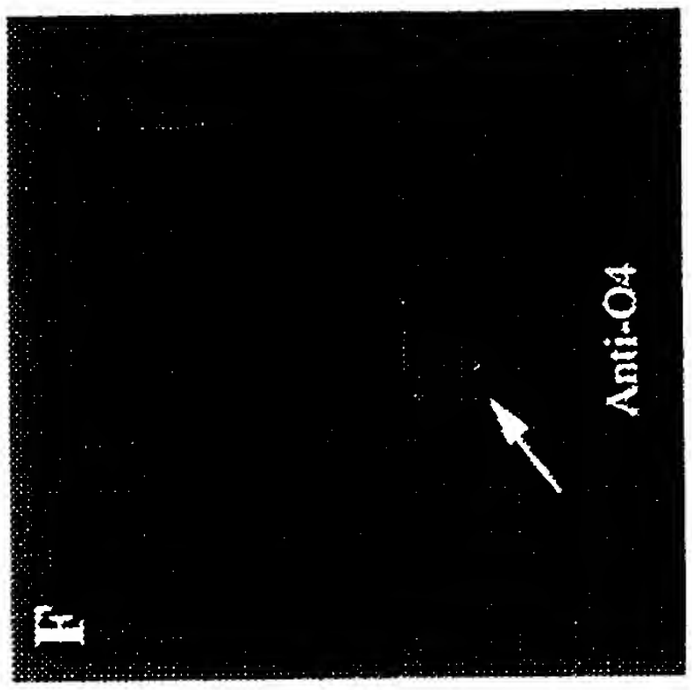


Fig. 4G

Fig. 4H

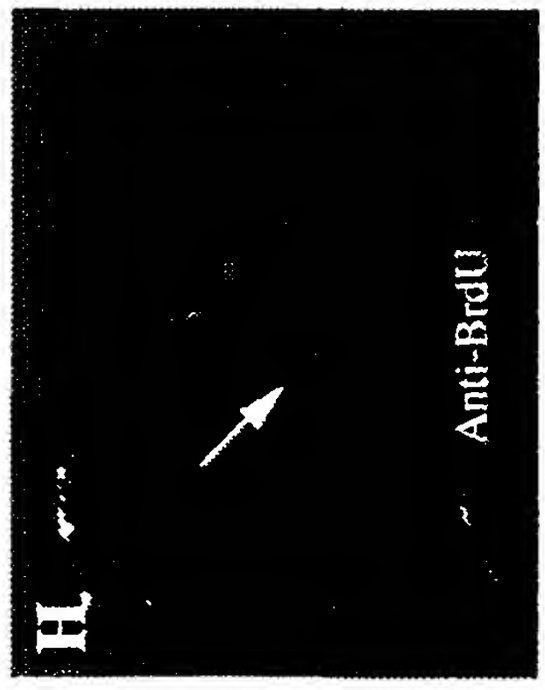


Fig. 4I

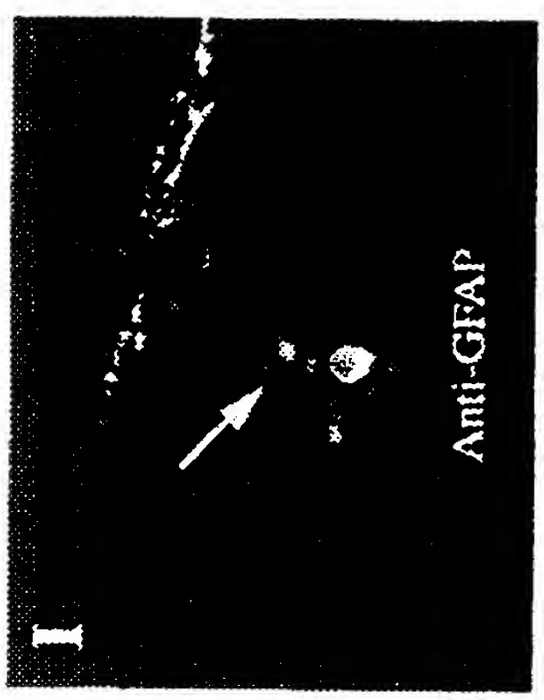


Figure 5

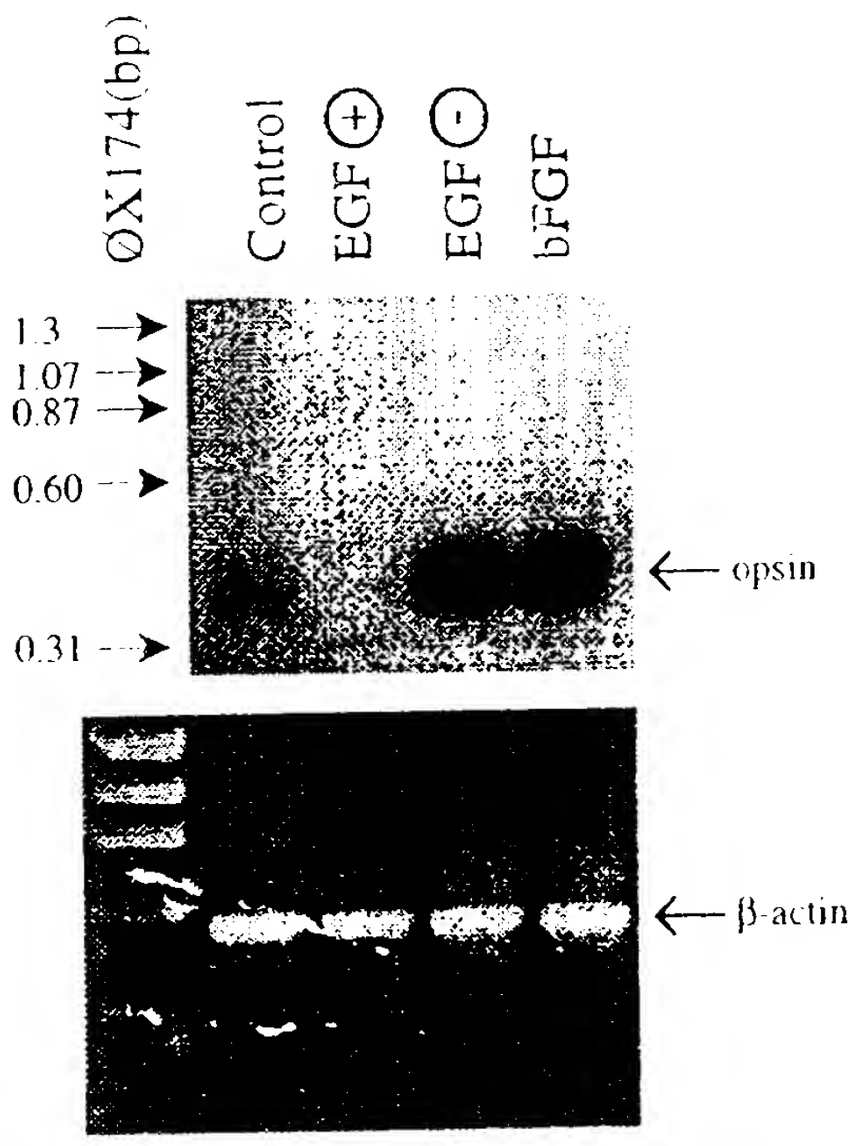


Fig. 6A

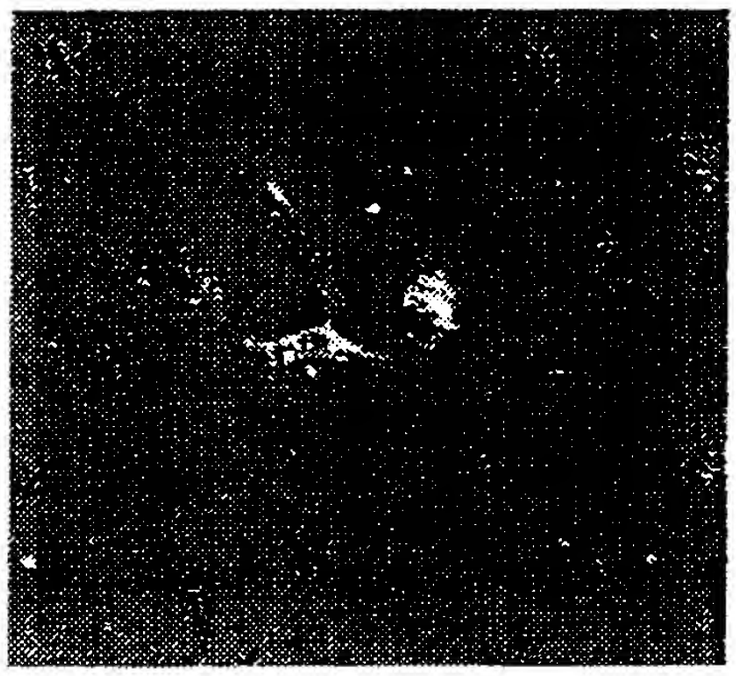
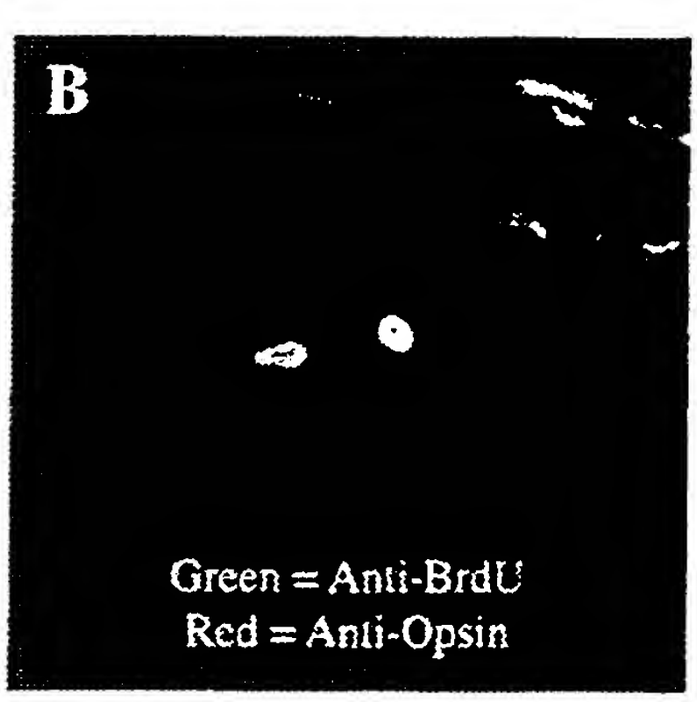


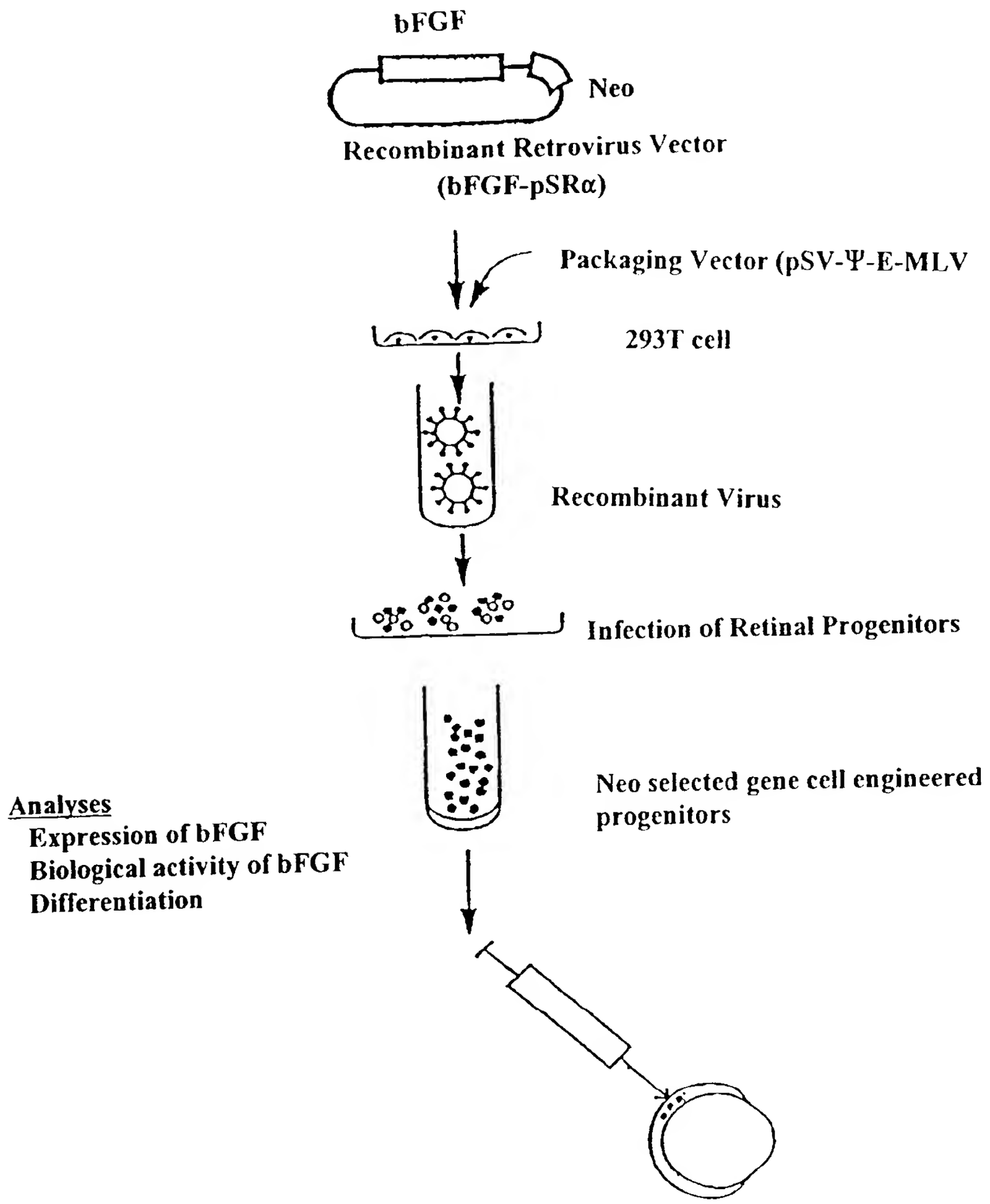
Fig. 6B



Embryonic Retina
Dissociation and Plating
2-3x10³ cells/cm²
⊕EGF
Dissociation
Replating
Clonal Analysis
1° Neurospheres
Long Term Passaging
2° Neurospheres
Cryopreserved Neurospheres
Re-Expansion
⊕BrdU
Dissociation
Differentiation
⊖EGF
⊕1% FBS
⊕Co Culture/Growth Factor
Immunocytochemical Analysis (Identification of BrDu⁺ Nestin⁺ Progenitors)
Immunocytochemical Analysis of Photoreceptor Differentiation (Identification of Brdu⁺ and Ret-P1⁺ cells)
BrdU Tagged Progenitors
Fast Blue Labeling
Retina
Periretinal Space
● = Progenitors
Morphological and Morphometric Analysis
Immunocytochemical Analysis
In Situ Hybridization Analyisis

Figure 7

Figure 8


bFGF
Neo
Recombinant Retrovirus Vector
(bFGF-pSRα)
Packaging Vector (pSV-Ψ-E-MLV
293T cell
Recombinant Virus
Infection of Retinal Progenitors
Neo selected gene cell engineered progenitors
Analyses
Expression of bFGF
Biological activity of bFGF
Differentiation
Sub-retinal transplantation of genetically engineered progenitors
Analyses
Morphological + morphometric
Immunocytochemical
In situ hybridization
Tunel

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/07377

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 5/00
US CL :435/325

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/325

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,416,260 A (KOLLER et al) 16 May 1995, see entire document.	1-20
A	US 5,453,357 A (HOGAN) 26 September 1995, see entire document.	1-20

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

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